Culture of Retinal Pigment Epithelium from Evisceration Specimens

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Human retinal pigment epithelial (RPE) cell cultures are usually obtained from donor eyes; isolation and culture of RPE cells obtained by evisceration has not been reported previously. The present study attempted to isolate and cultivate RPE cells from evisceration specimens obtained from two cases with severe ocular trauma. Two different isolation methods, explantation and enzymatic dissociation, were used. In Case 1, RPE cells grew from the explants, but were contaminated with other cells such as fibroblasts and melanocytes, and no pure RPE cultures were obtained by explantation. In Case 2, RPE cells were separated from choroids using 0.25% trypsin before plating for culture, which effectively eliminated contaminating cells. A pure RPE culture was obtained and cultured with F12 medium supplemented with 30% fetal bovine serum. With this enzymatic dissociation method, cultured RPE cells grew to confluence in primary culture and could be maintained in culture for five passages. Cultured RPE cells were identified by the presence of cytokeratin expression, as shown by immunocytochemical staining. These isolation and culture methods provide alternative sources for human RPE cells and could be useful in studies of the cell biology and pathophysiology of human RPE cells.

Key Words: retinal pigment epithelial cells, explantation, enzymatic dissociation
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Isolation and cultivation of human retinal pigment epithelium (RPE) was first established three decades ago. The major source of human RPE cells is cadaveric eyes [1–8], although some RPE cells have been obtained from preretinal membranes excised during vitrectomy surgery for proliferative vitreoretinopathy [9–11]. To date, no report has discussed RPE cell isolation from samples obtained during evisceration surgery. We report two patients who were blinded due to severe ocular trauma and who underwent evisceration surgery. Primary RPE cells were obtained from these patients by explantation and enzymatic dissociation. These isolation and culture methods provide alternative sources for human RPE cells and could be useful in studies of the cell biology and pathophysiology of human RPE cells.

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Materials and Methods

Reagents

F12 nutrient mixture, fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Life Technology (Grand Island, NY, USA). Antibodies against cytokeratin (mouse monoclonal antibody, AE1/AE3), S100 (rabbit polyclonal antibody), and vimentin (mouse monoclonal antibody, V9) were all purchased from Dako (Carpinteria, Denmark).

Explantation method

Case 1 was an 18-year-old male who lost vision due to trauma to the left eye and was primarily sutured. He underwent evisceration surgery 1 week later. The cornea

was removed by limbal excision and the lens was extracted. The iris and vitreous gel were also removed. The retinal and choroidal membranes were stored in F12 medium for cell culture. The membrane was cut into small pieces (1 × 1 mm) that were plated into a 35 mm Falcon dish (Becton Dickinson, Oxnard, CA, USA), with 0.5 mL F12 medium containing 20% FBS. Additional culture medium (0.5 mL) was added 1 to 2 hours later, and again after 24 hours (1 mL). Cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37°C. The media were renewed every 3 days until confluency.

Enzymatic dissociation method

Case 2 was a 45-year-old male who had lost vision in his right eye 19 years previously when it was pierced by a metal rod, and his eye gradually shrank. He underwent evisceration surgery as described for Case 1. The choroidal membrane was dipped in F12 medium and kept for further primary culture. The membrane was immersed in 0.25% trypsin solution (Gibco, Grand Island, NY, USA) and incubated at 37°C for 30 minutes until the RPE detached from the stroma. The isolated cells were collected by centrifugation and resuspended in culture medium (F12 medium containing 10% FBS). Cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37°C. The media were renewed every 3 days until confluency.

Immunocytochemical stain

Cultured cells were seeded on chamber slides for overnight incubation to detect intracellular antigen expression. Cells on slides were fixed with a mixture of acetone and methanol (1:1) at –20°C for at least 30 minutes. Following washes with phosphate-buffered saline (PBS), the cells were treated with 50 µL of primary antisera against cytokeratin, S100, and vimentin, respectively, at 1:50 dilution at room temperature for 1 hour. After washes with PBS, the immunoreactive signal was linked to secondary fluorescein isothiocyanate-conjugated goat anti-rabbit or anti-mouse immunoglobulin G antibodies (Chemicon, Temecula, CA, USA) at 1:1,000 dilution, and incubated at room temperature for 1 hour. The cells were finally washed five times with PBS, mounted in 10% glycerol in PBS, and observed under a fluorescence microscope.

Results

After incubation for 7 days, component cells from Case 1 were migrating out from and colonizing around the excised tissues. These outgrowing cells revealed heterogeneity in morphology, including pigmented epithelioid cells, unpigmented fibroblast-like cells, and pigmented cells with dendrites (Figure 1). Most were fibroblast-like cells, which are capable of propagating. The fibroblast-like cells outnumbered RPE cells after 10 days of culture, and reached confluency after 23 days of incubation.

Using the enzymatic dissociation method (Case 2), we observed a large quantity of pigmented cells attached to the culture dish after seeding overnight. The attached cells started to spread in polygonal or epithelioid shapes after 3 days of incubation. There was a round transparent zone in the cell nucleus and a large number of pigmented brownish granules in the cytoplasm. The attached cells started to grow after 5 days of incubation and the pigment content of each cell significantly decreased, possibly due to the dilution effect of mitosis (Figure 2). The cells reached confluence

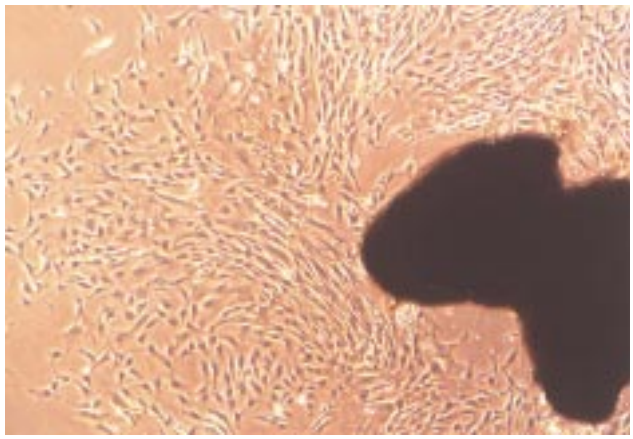

Figure 1. *Photomicrograph of primary retinal pigment epithelial cells obtained by the explantation method.*

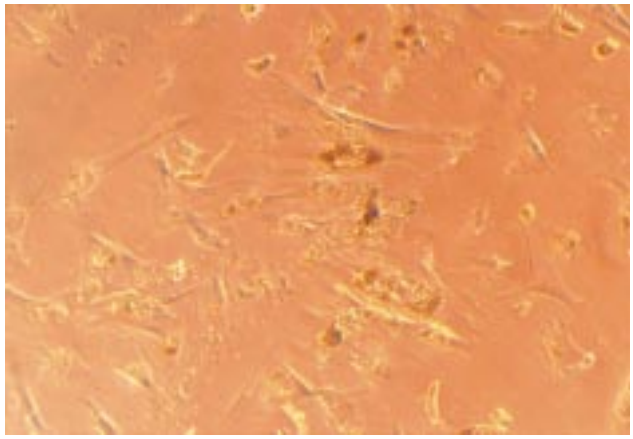

Figure 2. *Photomicrograph of primary retinal pigment epithelial cells obtained by the enzymatic dissociation method.*

after 15 days of incubation and were subcultured. The subcultured cells continued to grow and remained epithelioid in morphology, while the intracellular pigmented granules continued to decrease due to mitosis. The cells became senescent and the doubling time was longer than 5 days after six passages.

Immunocytochemical staining indicated that the cells from Case 2 in all passages were cytokeratin-positive (Figure 3). The positivity percentage of all staining was higher than 90% of isolated cells (data not shown). They all displayed characteristics typical of epithelial cells.

Discussion

RPE plays multiple physiologic roles in the retina, such as phagocytosis of photoreceptor outer segment, metabolism of vitamin A, regulation of transportation of water and electrolytes, and production of various growth factors, neurotrophic factors, and cytokines [6–8,12–15]. Melanin molecules in RPE, serving as anti-oxidant agents and free-radical scavengers, can absorb visible light and block the deleterious effects of ultraviolet light [16]. In addition, RPE plays an important role in maintaining the physiologic functions of the retina, exchanging metabolites through the choroidal blood circulation. Pathogenic alteration in RPE cells is strongly associated with several ocular diseases, such as age-related macular degeneration, proliferative vitreoretinopathy, Usher syndrome type 1B, and Sorsby macular dystrophy [17–19].

The isolation and culture of human RPE cells are important in the study of cell biology and pathophysiology. In this paper, we report two cases in which tissues from evisceration surgery were used to culture primary RPE cells. We chose evisceration specimens because they are the most accessible sources of human RPE cells for clinical ophthalmologists. In the first case, it proved difficult to obtain pure RPE cells using the explantation method due to the fact that the choroidal membrane contains different types of cells, including melanocytes, fibroblasts, vascular endothelial cells, and pericytes. Flood et al [2] and Newsome [5], in their retrospective history of RPE cell culture, indicated that many investigators have tried to isolate RPE cells using the explantation method but failed. Primary RPE cell culture with a high degree of purity was impossible until Mannagh et al proposed enzymatic digestion as an isolation method [1]. Since then, enzymatic microdissection has become, without exception, the most common method of isolating RPE from cadaveric eyes [2–8].

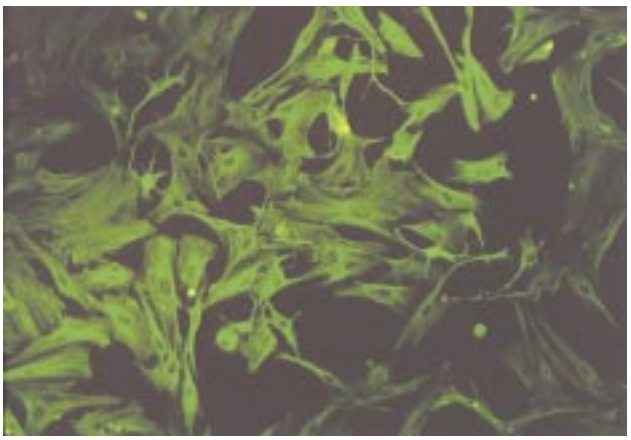

Figure 3. *Immunocytochemical detection of cytokeratin expression.*

We therefore used the enzymatic dissociation method in Case 2. It appeared that some, if not all, of the RPE cells could be successfully separated from choroidal membranes, and that the enzymatic treatment was optimized so as not to hamper cell viability, so that isolated cells were able to attach, spread, and proliferate after inoculation to culture dishes. The morphology of the attached cells was apparently different from that of fibroblasts, in that they contained high amounts of pigment granules in the cytoplasm. Since adult RPE cells lack *de novo* melanogenesis, the intracellular content of pigment granules becomes increasingly diluted throughout the progression of cellular mitosis, particularly when they are isolated and maintained *ex vivo*. Nevertheless, cell morphology remained the same as the typical pattern of epithelium, and cells continued to grow for at least five passages. The expression of cytokeratins is considered the most reliable differentiation marker for the epithelium, and should not be present in fibroblasts and melanocytes. The cells cultivated in Case 2 were definitely RPE, as confirmed by positive staining for cytokeratin.

In summary, we successfully isolated primary RPE cells using the enzymatic digestion method from a patient who had undergone evisceration surgery. This is the first case in which human RPE cells were cultured from non-cadaveric materials. Compared with the explantation method, the enzymatic dissociation method is the better one for retrieving experimental materials from clinical subjects, in terms of efficiency and cell purity.

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採用眼球剜除術後標本所培養出之視網膜色素上皮細胞

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過去尚未有以眼球剜除術後標本試做人類視網膜色素上皮細胞分離和培養之報告。本研究利用兩個嚴重眼球外傷的個案取其眼球剜除之標本進行視網膜色素上皮細胞之培養。我們採用兩種不同的細胞分離方法。個案一採傳統組織體外培養法，結果發現雖然視網膜色素上皮細胞順利生長，但是被纖維母細胞，黑色素細胞等其他種類細胞污染而駁雜不純。個案二採用酵素分離法，先使用 0.25% 胰蛋白 將視網膜色素上皮細胞自脈絡膜取下再移至培養皿即可成功抑制其他細胞之污染。得到純種視網膜色素上皮細胞之後採用 F12 培養基加入 30% 胎牛血清可將視網膜色素上皮細胞繼續繁衍至五個世代。此視網膜色素上皮細胞經採用免疫細胞染色法證實確含有上皮細胞特有之細胞角質素無誤。本研究中的細胞分離及培養方法提供了人類視網膜色素上皮細胞培養的另一選擇，並且可對視網膜色素上皮細胞的細胞生物學及病理生理學研究有所裨益。

關鍵詞：視網膜色素上皮細胞，組織體外培養，酵素分離法

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